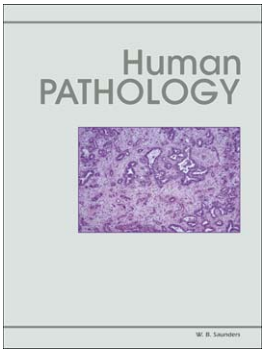

Inflammatory cell infiltrates in advanced metastatic uveal melanoma

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Title Page

Inflammatory cell infiltrates in advanced metastatic uveal melanoma

Short title: Inflammation in advanced metastatic uveal melanoma

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ABSTRACT

Current treatments for metastatic uveal melanoma (mUM) are limited and rarely prolong patient survival. Immunotherapy trials for mUM are few, and to-date have demonstrated only marginal success. High densities of tumour-associated-macrophages (TAMs) and infiltrating T-lymphocytes (TILs) in primary UM are associated with poor prognosis. Little is known about the immune microenvironment of mUM. Our aim was to examine the presence and distribution of TAMs and TILs in mUM within the liver. Whole tissue-sections of liver mUM (n=35) were examined by immunohistochemistry. For TAMs, monoclonal-antibodies (mAbs) against CD68 and CD163 were used. Macrophage density and morphology were scored using previous established systems. Density and spatial-distribution of TILs were highlighted using Abs against CD3 (pan-lymphocyte marker), CD4 (T-helper cells) and CD8 (T-cytotoxic cells). CD68+ and CD163+ TAMs were seen within the tumour in all 35 specimens; their density was 'moderate' in 50% of cases, 'few' in 43% and the majority showed an 'indeterminate' phenotype. CD3+ TILs were noted both within mUMs and surrounding the tumour. Of these CD8+ TILs were 'few' in number within mUM but were predominantly seen peri-tumourally at the tumour/normal liver interface, whilst CD4+ TILs showed a high perivascular density within mUM. CD68+ and CD163+ TAMs of 'indeterminate' morphology were observed in mUM, suggesting a tendency towards the pro-tumourigenic M2-phenotype. CD4+ TILs were seen within the mUM, whereas CD8+ TILs tended to be peri-tumoural. The biological and functional roles of inflammatory cells in mUM requires further investigation, to determine if they represent potential targets for future therapies in mUM.

Keywords: Metastatic uveal melanoma, inflammation, macrophages, T-cells, liver, immunotherapy.

INTRODUCTION

Uveal melanoma (UM) is an aggressive intraocular malignancy with up to 50% of UM patients developing metastatic disease, usually involving the liver, even several years after the primary treatment [1-4]. Current treatments for metastatic UM (mUM) to the liver include: metastectomy, liver resection, radio- and/or chemotherapy, radiofrequency ablation; all of these therapies are limited, only being suitable for certain patients, and rarely prolonging patient survival [3-6]. Consequently, there is an urgent need to improve current treatments for established metastatic disease alongside adjuvant therapy.

Chronic inflammation is recognized as a hallmark of cancer, and is thought to be a key mediator in all steps of tumourigenesis – from initiation, through to progression and metastasis [7-11]. This has recently led to the development of new treatment strategies using immunotherapies, such as: ipilimumab, nivolumab and pembrolizumab, which have been successful in subsets of patients with metastatic cutaneous melanoma. However, ipilimumab has only shown marginal success in mUM to date [4, 12-18].

It is known that high densities of tumour-associated-macrophages (TAMs) and infiltrating T-lymphocytes (TILs) in primary UM are found in tumours with a 'high risk' of metastasising. These include UM with a large basal diameter, predominantly of epithelioid cell-type, high microvascular density, and monosomy 3 [2-4, 19-24]. Macrophages and T-cells are central to the general regulation of the immune response. TAMs have been implicated in tumourigenesis by promoting angiogenesis, tumour cell migration and invasion, and tumour growth [7-11, 13-14]. T-cells, in contrast, orchestrate the immune response to cancer through recognition, priming and attacking cancer cells [25-29]. Very little is known about the immunomodulatory microenvironment of mUM, and studying this is difficult as mUM specimens are usually difficult to acquire, and often are small percutaneous biopsies.

In this study we examined the density and spatial distribution of TAMs and TILs in advanced cases of hepatic mUM.

MATERIALS AND METHODS

The study was approved by the Health Research Authority (REC Ref 11/NW/0759) and conducted in accordance with the Declaration of Helsinki. All samples were provided by the Ocular Oncology Biobank (REC Ref 16/NW/0380) and the Liverpool Bio-Innovation Hub following approval from their Biobank.

Specimens: Archival formalin-fixed paraffin embedded (FFPE) specimens of mUM were obtained from: 1) 19 patients who had undergone local resection of their liver metastases, and 7 patients who had percutaneous fine needle biopsies at Aintree University Hospital between 2005 and 2016; and 2) dissection of the liver metastases during the autopsy of 9 patients (Queen Alexandra Hospital Portsmouth). All of the samples were assessed by a Senior Consultant Histopathologist (SEC), and the hepatic metastases classified for the following: staging, dominant cell type, degree of pigmentation, presence of necrosis and growth pattern.

Immunohistochemistry (IHC): Sections were cut at 4µm from the FFPE blocks and processed for IHC as previously described [30]. Briefly, antigen retrieval and IHC were performed using the Dako PT Link and Autostainer Plus with the EnVision™ FLEX+ detection system according to the manufacturers' recommendations (Dako U.K. Ltd., Cambridgeshire, U.K.).

Primary antibodies (Abs) against CD68 (mouse anti-human PG-M1, M0876; Dako, Cambridge, U.K.) at 1:200 and CD163 (mouse anti-human, NCL-L-CD163; Leica Biosystems, Newcastle Upon Tyne, U.K.) at 1:400 were used to identify the macrophages. Immunostaining of T-cells was

undertaken using the following primary Abs: pan T-cell marker CD3 (polyclonal rabbit anti-human, ready to use, IR503; Dako, Cambridge, U.K.); helper T-cell marker CD4 (monoclonal mouse anti-human, NCL-L-CD4-368; Leica Biosystems, Newcastle Upon Tyne, U.K.) at 1:20 and cytotoxic T-cell marker CD8 (monoclonal mouse anti-human, M7103; Dako, Cambridge, U.K.) at 1:200. Positive and negative controls for each of the primary antibodies were run for each assay.

Positive staining was detected with either: 3-amino-9-ethylcarbazole (AEC peroxidase substrate, SK-4200; Vector® Laboratories Ltd., Peterborough, U.K. for 30 minutes) or 3,3'-diaminobenzidine (DAB+ chromogen, K3467; Dako, Cambridge, U.K. for 20 minutes). Slides were counterstained with Mayer's haematoxylin and mounted with an aqueous or resin-based mountant, respectively.

Scoring of TAMs and TILs within the liver metastases: Following the grading system previously described by Mäkitie et al.[22] TAM and TIL density was qualitatively scored as 'few,' 'moderate' or 'many.' For TAMs, their morphology was also assessed and the predominant cell type was recorded as: 'round,' 'dendritic,' or as 'indeterminate' (in between the dendritic and round morphologies).

Slides were subsequently scored by 3 independent observers (YK, CM and HK) and the number, spatial distribution and/or morphology was recorded for TAMs and TILs within the liver metastases. The entire specimen was evaluated using a high power field (x40). Immunopositive cells in areas of necrosis or normal liver tissue distant from the mUM were not included in the scoring. Discrepancies in scoring were resolved by consensus between all members involved during re-analysis.

RESULTS

Patients and samples: Detailed clinical, pathological and genetic data for liver metastases cases 1 to 7 have been previously published by our group [30]. The growth patterns of mUM to the liver from sections obtained post-mortem (cases 8 to 16) have also been described [31]. For the current study, these 16 specimens from both studies and an additional 19 mUM specimens (cases 17 to 35) were examined for their inflammatory cell infiltrates. A total of 35 hepatic metastases were thus included.

There were 17 males and 14 females (the gender of 4 autopsy cases was unrecorded). Age at primary management was available for 26/35 cases (Table 1), with a median of 60.5 years (range: 38 – 78 years). All patients had died from advanced and widespread mUM. All but 1 case had stage 3 metastases as previously reported [32]. Histological examination showed the liver metastases to be of epithelioid cell type in 28 cases and spindle cell type in 7; with an infiltrative growth pattern in 21 cases and a nodular growth pattern in 14. Degree of pigmentation ranged from: heavy in 6 cases; moderate in 11; mild in 6 and none in 12 cases. Fourteen out of the 35 cases had areas of necrosis (Table 1).

Scoring of TAMs and TILs within the liver metastases: The mean interobserver agreement was 88% for scoring the number/density of TAMs and TILs, and 85% for evaluating the TAM morphology. Density of TAMs and TILs within the mUMs was scored as shown by the representative panels in Fig. 1. TAM morphology was assessed as shown in Fig. 2.

For TAMs, CD68+ cell infiltrates were scored as ‘moderate’ in 18 cases (51%) and ‘few’ in 13 cases (37%). CD163+ cell infiltrates were evaluated as ‘moderate’ in 17 cases (49%) and ‘few’ in 17 cases (49%) (Table 2). TAMs of ‘indeterminate’ phenotype dominated in 16/35 (46%) mUM; 13 cases (37%) showed a ‘dendritic’ phenotype; 5 cases (14%) had equal numbers of both ‘round’

and ‘dendritic’ morphologies (i.e. neither cell morphology dominated); and 1/35 (3%) demonstrated a ‘round’ phenotype. Interestingly, ‘many’ dendritic macrophages were observed at the interface of normal liver and tumour tissue. These were not, however, included in the scoring.

For TILs, CD3+ lymphocyte infiltrates scored as ‘moderate’ in 12 samples (34%), ‘and ‘few’ in 22 cases (63%) (Table 2). Diffuse scattered CD3+ cells were noted within the metastases (Fig. 3), with ‘many’ also observed at the normal/tumour interface, for all 35 cases. CD4+ cells were seen in all cases (Fig. 3) with evident peri-portal and perivascular aggregations (Fig. 4A). Densities were evaluated as ‘moderate’ in 4 specimens (11%) and ‘few’ in 31 cases (89%). For CD8+ cells, the densities were scored as ‘moderate’ in 6 samples (17%) and ‘few’ in 29 cases (83%) (Table 2). Interestingly, aggregates or heavy CD8+ cell infiltrates were observed circumferentially at the edge of the tumour (Fig. 4A); whereas only ‘few’ CD8+ cells were seen within the liver metastases in most metastases. In the cases scored as ‘moderate,’ these aggregates of circumferential CD8+ cells were noted as present within tumours in multiple areas, and hence included in the scoring.

In some cases, isolated and single mUM cells were observed distant from the main metastatic deposit: these were rarely associated with an inflammatory response (Fig. 4B).

DISCUSSION

In this study we present novel data describing the inflammatory cell infiltrates within and surrounding hepatic mUM. Both CD68+ and CD163+ TAMs of an ‘indeterminate’ morphology were the dominant subtype in all mUM, although a significant number of cases were also associated with macrophages of ‘dendritic’ phenotype. CD4+ TILs were seen in a perivascular distribution within the mUM, whereas CD8+ lymphocytes were mainly peri-tumoural with only occasional cells seen within the metastatic tumour masses.

Macrophages and T-cells are central regulators of inflammation and the immune response [8-11]. Inflammation is a hallmark of cancer and thought to be a key mediator in all steps of tumourigenesis and tumour spread [7-11]. Under the influence of various chemokines and cytokines, circulating monocytes and tissue resident macrophages polarise to either the: a) *round*, classically activated, pro-inflammatory, tumour lytic M1 phenotype; or b) the elongated *dendritic*, alternative pathway, pro-angiogenic and pro-tumourigenic M2 TAM phenotype [25, 27, 29, 33-34]. There is much controversy in the literature regarding the classification of M1 and M2 TAMs, since they were first described in the mouse, and it is difficult to determine how much can be extrapolated to human macrophage subtypes. Indeed, the classification of TAMs and their markers is far more complex with their further overlapping subcategories and associated plasticity. That being said, it is generally agreed that that CD68+ CD163- macrophages with round morphology represent the M1 phenotype, and dendritiform CD68+ CD163+ cells correspond to M2 [23-29, 33-37]. High densities of M2 TAMs are associated with a poor prognosis in various primary cancers, including cutaneous melanomas, ovarian-, pancreatic-, breast-, and colorectal carcinomas, and indeed primary UM [33-37].

In the present study both CD68+ and CD163+ TAMs were seen at similar densities in all hepatic mUM samples analysed, suggesting that at least three quarters of macrophages within mUM were of the M2 subtype. These CD68+ CD163+ TAMs were predominantly of an ‘indeterminate’ and ‘dendritic’ morphology; and were mainly seen within the metastatic tumour masses. This was also reported by Mäkitie et al. in primary UM [22]. The dendritic and indeterminate/intermediate TAM morphology further indicates polarisation to a M2 subtype/intermediary. It remains unknown, however, whether these TAMs are hepatic resident macrophages (i.e. the Kupffer cells) entrapped within the mUMs, or inflammatory macrophages that have responded to stimuli, or possibly macrophages that ‘accompanied’ UM cells during the process of entire metastasis. Macrophage labelling studies in preclinical models are required to answer these questions.

Importantly, we also provide novel information regarding the density and distribution of T-cells in advanced mUM. Only a 'few' CD4+ T-cells were present within the mUMs, although numerous perivascular aggregates were noted. In contrast, CD8+ cytotoxic/killer T-cells predominantly encircled the entire metastatic deposit. This would suggest that CD4+ T-cells and, in particular, CD8+ cells are not able to infiltrate the tumour mass, and thus the mUM cells are 'protected' from immune attack. Such 'immune evasion/exclusion,' has been reported in other solid primary cancers [9-11, 35-36]. The ability of cancers to evade immune recognition may be explained by a number of factors, including: a) their genetic instability giving rise to certain tumour cells with poor immunogenicity with loss of major histocompatibility complex glycoproteins; b) the overexpression of oncogene-coded proteins; or c) alteration in the processing of antigenic peptides. These factors would prevent effective immunosurveillance/recognition of the tumour antigens by T-cells, and consequently loss of priming of an immune response by the helper T-cells (CD4+) and immune attack by cytotoxic T-cells (CD8+) [9-11, 35-36]. The failure of these two critical immune functions: direct priming of an immune response and T-cell mediated cytotoxicity, may in part explain the successful colonisation process of mUM. Interestingly, we also observed isolated mUM cells scattered within the parenchyma with no associated inflammatory response, in some cases quite distant from the larger metastatic deposits. It is unclear whether these isolated cells have seeded from the main hepatic metastases or represent single isolated mUM within a larger pool of disseminated tumour cells from the primary UM.

The authors recognise that these are preliminary observations of the advanced metastatic environment in a fairly limited number of rare mUM specimens. Further descriptive studies in a much larger cohort including less advanced/early staged cases of mUM (though these may be difficult to detect with current imaging modalities), as well as functional studies, are required to fully define the role of inflammation in mUM. Furthermore, we are aware that the CD4+ immunostaining may also have highlighted some of the liver's resident stern Kupffer cells in

addition to T-cells. However, in this regard, caution was taken in the assessment of all specimens by the 3 independent observers and any discrepancies were resolved by collaborative consensus. Finally, the authors are aware that functional analyses of the macrophages within the mUM would be able to determine the degree of their activation and whether there is an active suppression of their function by the tumour cells.

By better understanding of the mUM microenvironment and inflammatory cell infiltrates, innovative and effective adjuvant immunotherapies against metastatic disease may be identified to: augment the anti-tumour T-cell mechanisms perhaps in combination with immune checkpoint targeted therapies [9-12, 15, 38], increase immune infiltration or increase/switch polarisation of TAMs from the M2-type to the pro-inflammatory, anti-tumourigenic M1 phenotype. Future collaborative studies will also examine inhibitory immune checkpoints, such as: cytotoxic T-lymphocyte antigen-4 (CTLA4), programmed cell death protein-1 (PD-1) and its ligand (PD-L1).

In summary, we have shown CD68+ CD163+ macrophages of an ‘indeterminate’ and ‘dendritic’ morphology in advanced mUM, suggesting tendency towards the pro-tumourigenic M2 phenotype. Furthermore, CD4+ TILs were noted in a perivascular distribution within the mUM whereas CD8+ TILs were mainly peri-tumoural with only ‘few’ actually infiltrating the tumour. Further studies into the early stages of mUM, are required to better understand the association of inflammatory cell infiltrates with mUM cells, and if they represent potential targets for adjuvant immunotherapies.

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Author Contribution: Each author made substantial contributions to the design, acquisition, analysis and interpretation of data for the work in this study. Each author also made substantial contribution in: drafting; revising and approval of the final version of the manuscript to be published.

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Figure Legends:

Fig. 1: Scoring system developed for assessing the density of TAMs and TILs within hepatic mUM (3-amino-9-ethylcarbazole [AEC peroxidase substrate]; magnification x40).

Fig. 2: The morphological types of TAMs (indicated by arrows; AEC peroxidase substrate; magnification x40). A) Dendritic; B) Round.

Fig. 3: TILs density within the hepatic mUM (3,3'-diaminobenzidine [DAB]; magnification x40).

Fig. 4: A) The spatial distribution of TILs within the hepatic metastases (DAB). Aggregates of CD4+ T-cells (indicated by black arrow) were seen in a perivascular distribution, whereas CD8+ T-cells (indicated by black arrow) were seen at the interface of tumour (T) and normal liver (N). B) Isolated distant mUM cells (indicated by black arrow) observed with no surrounding inflammatory cell infiltrate (H&E and corresponding Melan A; magnification x20).

Fig. 1

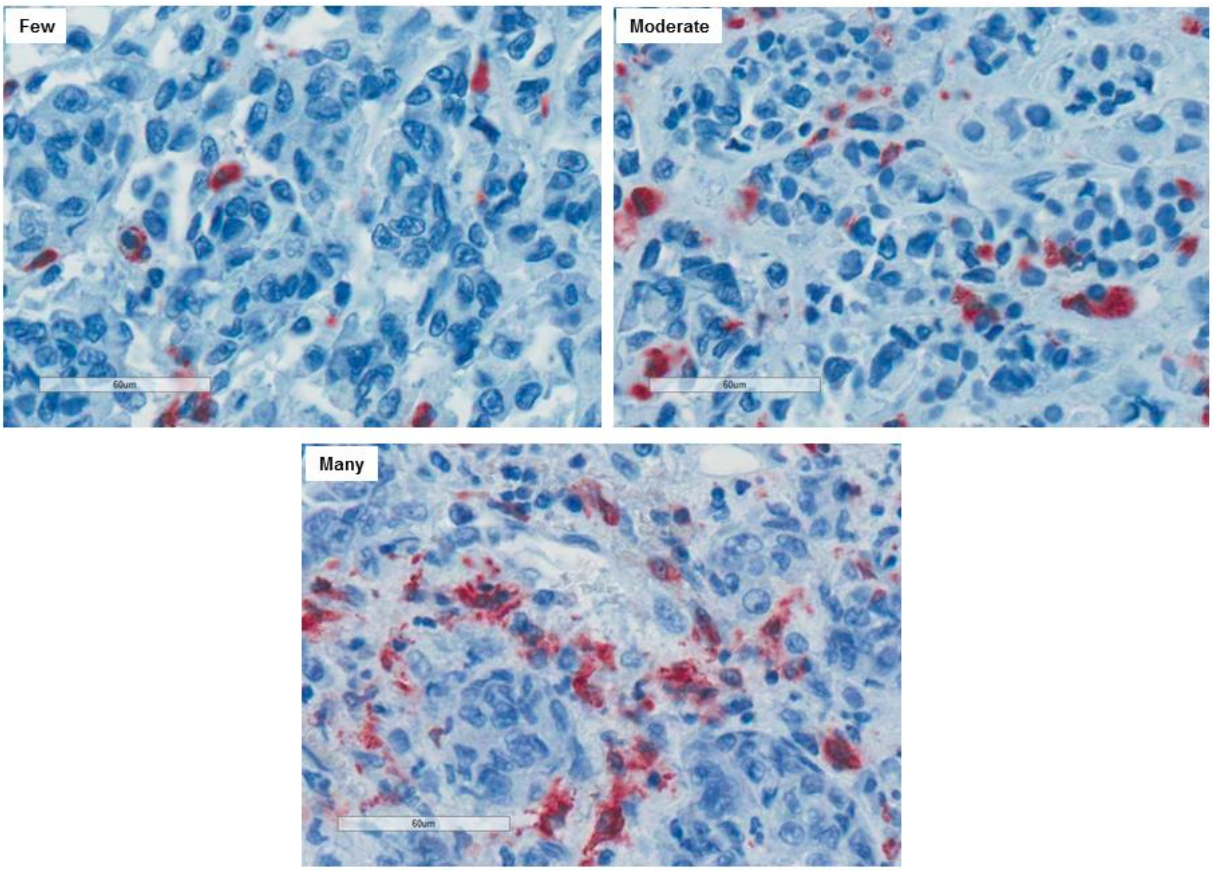

Fig. 2

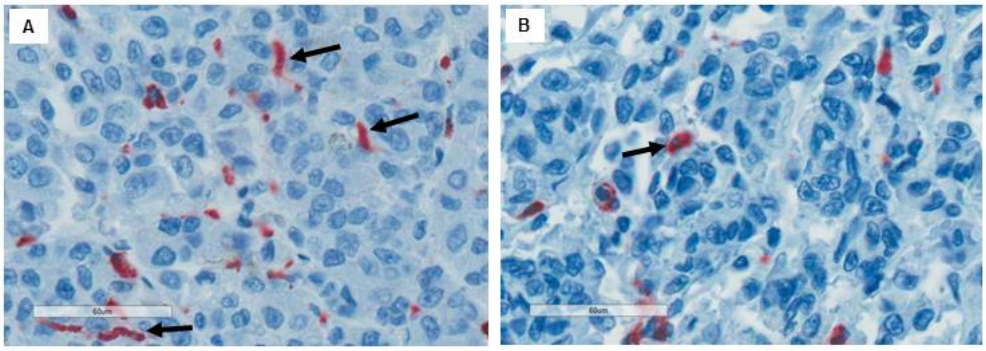

Fig. 3

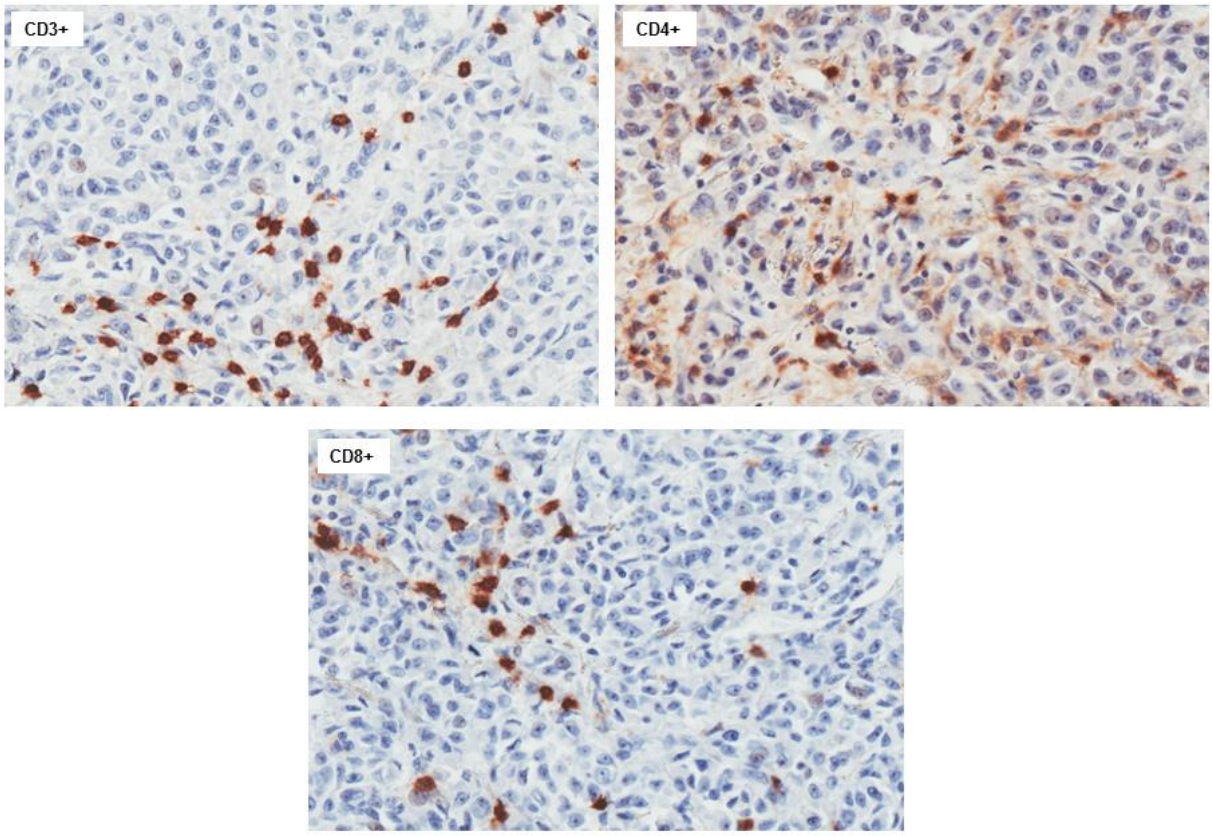

Fig. 4

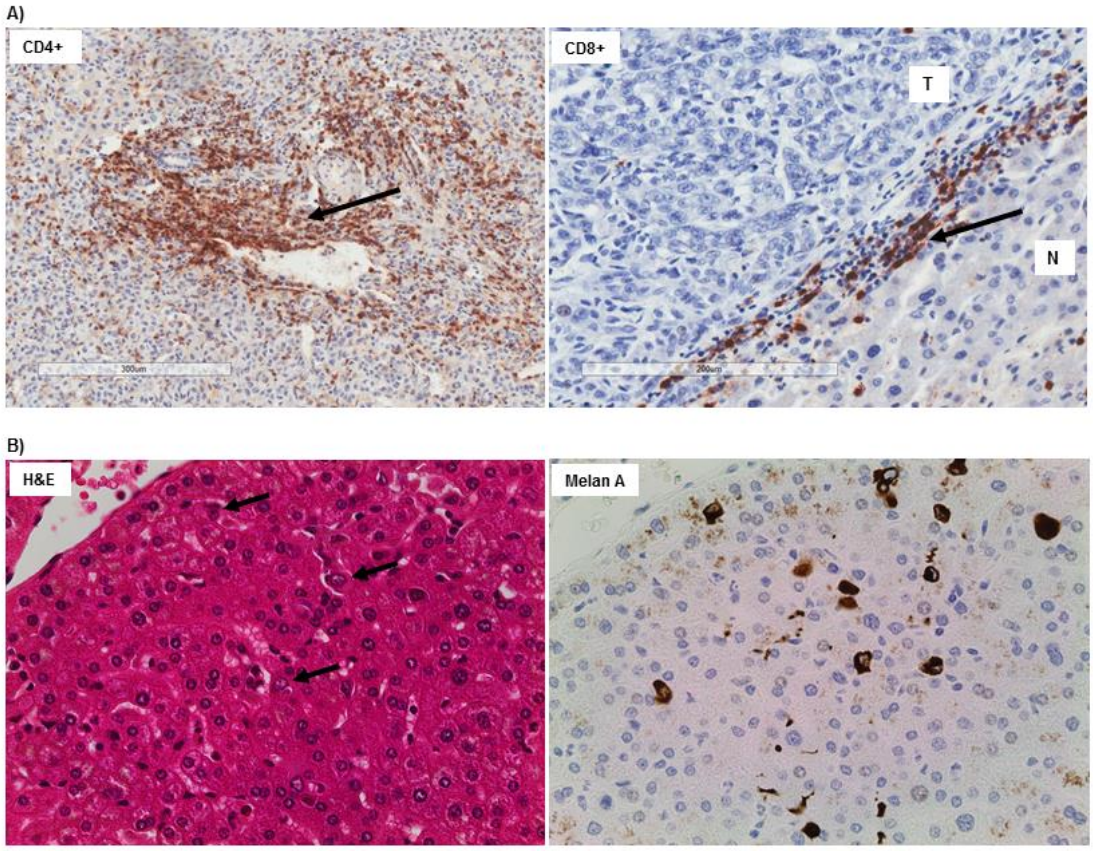

Table 1: Clinical and histopathological data from the 35 cases of mUM analysed.

Case No.	Gender	Age at PM	Sample type	Stage*	Dominant cell type	Degree of Pigmentation	Necrosis	Growth pattern (nodular/infiltrative)
1	M	64	Resection	3	Epithelioid	None	N	Infiltrative
2	F	39	Resection	2	Epithelioid	Mild	N	Nodular
3	F	69	Resection	3	Epithelioid	None	Y	Infiltrative
4	F	64	Resection	3	Epithelioid	Mild	Y	Infiltrative
5	M	66	Resection	3	Epithelioid	None	Y	Nodular
6	M	54	Resection	3	Spindle	Moderate	Y	Infiltrative
7	M	67	Resection	3	Epithelioid	None	Y	Infiltrative
8	M	75	Autopsy	3	Epithelioid	Moderate	Y	Nodular
9	M	NR	Autopsy	3	Epithelioid	Moderate	N	Infiltrative
10	F	NR	Autopsy	3	Epithelioid	Heavy	N	Infiltrative
11	M	NR	Autopsy	3	Epithelioid	Moderate	N	Infiltrative
12	F	62	Autopsy	3	Epithelioid	Mild	Y	Infiltrative
13	M	64	Autopsy	3	Epithelioid	Heavy	N	Infiltrative
14	F	NR	Autopsy	3	Spindle	Moderate	Y	Nodular
15	M	66	Autopsy	3	Epithelioid	Moderate	Y	Infiltrative
16	M	NR	Autopsy	3	Spindle	Focal heavy	Y	Infiltrative
17	M	63	Resection	3	Epithelioid	Mild	N	Infiltrative
18	F	57	Resection	3	Epithelioid	None	N	Nodular
19	F	56	Resection	3	Spindle	Moderate	Y	Nodular
20	F	38	Resection	3	Epithelioid	None	Y	Nodular
21	NR	NR	Resection	3	Epithelioid	None	N	Nodular
22	NR	NR	Resection	3	Epithelioid	Heavy	N	Infiltrative
23	NR	NR	Resection	3	Epithelioid	None	N	Infiltrative
24	M	45	Resection	3	Epithelioid	Heavy	N	Infiltrative
25	F	54	Resection	3	Spindle	Moderate	N	Infiltrative
26	F	53	Resection	3	Epithelioid	Mild	N	Nodular
27	M	46	Resection	3	Spindle	None	N	Nodular
28	M	39	Resection	3	Spindle	None	N	Nodular
29	F	78	Biopsy	3	Epithelioid	Mild	N	Nodular
30	NR	NR	Biopsy	3	Epithelioid	Moderate	N	Infiltrative
31	M	47	Biopsy	3	Epithelioid	Moderate	N	Infiltrative

32	F	52	Needle biopsy	3	Epithelioid	None	N	Nodular
33	F	74	Needle biopsy	3	Epithelioid	Moderate	N	Nodular
34	M	59	Needle biopsy	3	Epithelioid	Heavy	Y	Infiltrative
35	M	71	Needle biopsy	3	Epithelioid	None	Y	Infiltrative

Abbreviations: F = female; M = male; PM = primary management; NR = not recorded; N = no; Y = yes. *Staging according to Grossniklaus HE [32].

Table 2: Density of TAMs and TILs within the liver metastases of the 35 mUM cases analysed.

	TAMs		TILs		
Case No.	**CD68+**	**CD163+**	**CD3+**	**CD4+**	**CD8+**
1	Moderate	Few	Moderate	Moderate	Moderate
2	Moderate	Few	Moderate	Few	Moderate
3	Few	Few	Few	Few	Few
4	Few	Few	Few	Moderate	Few
5	Few	Few	Few	Few	Few
6	Few	Few	Few	Few	Few
7	Few	Moderate	Moderate	Few	Few
8	Moderate	Moderate	Few	Few	Few
9	Moderate	Moderate	Moderate	Few	Few
10	Moderate	Moderate	Few	Few	Few
11	Few	Few	Few	Few	Few
12	Few	Few	Few	Few	Few
13	Moderate	Moderate	Few	Few	Few
14	Few	Few	Few	Few	Few
15	Few	Few	Few	Moderate	Few
16	Few	Few	Moderate	Moderate	Few
17	Moderate	Moderate	Moderate	Few	Moderate
18	Moderate	Moderate	Moderate	Few	Few
19	Many	Many	Few	Few	Few
20	Moderate	Few	Few	Few	Few
21	Many	Moderate	Moderate	Few	Moderate
22	Moderate	Moderate	Few	Few	Few
23	Few	Few	Few	Few	Few
24	Moderate	Few	Moderate	Few	Few
25	Moderate	Moderate	Few	Few	Few
26	Many	Moderate	Few	Few	Few
27	Moderate	Few	Few	Few	Few
28	Few	Few	Few	Few	Few
29	Moderate	Moderate	Few	Few	Few
30	Moderate	Moderate	Moderate	Few	Few
31	Moderate	Few	Few	Few	Few
32	Few	Moderate	Moderate	Few	Moderate
33	Moderate	Moderate	Moderate	Few	Few
34	Moderate	Moderate	Few	Few	Few
35	Many	Moderate	Many	Few	Moderate

Highlights

- Current treatments for metastatic uveal melanoma (mUM) are limited.
- Little is known about the immune microenvironment of mUM.
- M2 tumour-associated-macrophages (TAMs) CD68+ and CD163+ were observed in mUM.
- CD4+ T-lymphocytes (TILs) were seen within mUM; CD8+ TILs were predominantly peri-tumoural. Functional studies are required.
- TAMs and TILs in mUM may be potential targets for future immunotherapies.